Determination of Connective-Tissue Components in Beef Using Simultaneous Equations Based on Amino-Acid Analyses

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ABSTRACT

Collagen, elastin and residual actomyosin in connective tissue of some lower grades of beef were determined from the contents of hydroxyproline, valine and glutamic acid, using three simultaneous equations based on amounts of these amino acids found in the pure proteins. The complete amino-acid profiles of the connective tissues agreed with the calculated protein compositions. Connective tissues were removed from the meat by dissection, and sequential saline extractions removed sarcoplasmic and myofibrillar proteins. Glycoproteins were removed by extraction with half-saturated calcium hydroxide; collagen, by autoclaving. Changes in composition after extraction or autoclaving were confirmed by histological investigations.

INTRODUCTION

IN RESEARCH on muscle meat the need frequently arises for a simple way to determine the main protein components. Histological staining and microscopy have indicated variability in the ratios of such components. Forrest et al. (1975) identified ground substance, collagen, elastin, and reticulin. Bailey and Sims (1977) found Collagens I, III and IV in the connective tissue of meats, and immunospecific methods have indicated that reticulin may be identifiable as Type III collagen (Gay, 1978). Light and Champion (1984, 1985) found collagens IV and V in perimysium and endomysium muscle fibers. Partridge (1962) and Mellon et al. (1967) have isolated and determined the amino acid content of elastin from beef ligamentum nuchae. The ground substance contained glycoproteins consisting of protein complexes with chondroitin sulfate and with hyaluronic acid (Merkel, 1978). Sacks et al. (1988) selectively degraded the protein and polysaccharide components in muscle connective tissue and determined their effects on the viscoelasticity of muscle.

Zarkadas et al. (1988) proposed protein analysis of connective tissue by determining unique amino acids with special chromatographic columns. Our objective was to describe a simpler method of analysis, requiring only a conventional amino-acid analyzer. Dissolution of soluble proteins from isolated connective tissues of chuck and round left the insoluble residue of actomyosin, collagen and elastin, which make up 96+% of the connective tissue, prior to autoclaving. All the hydroxyproline, valine, and glutamic acid found were presumed to come from residual actomyosin, collagen or elastin. We could therefore obtain three simultaneous equations using amino acids found in the three unknown proteins actomyosin, collagen and elastin. The various components—glycoprotein, collagen, and elastin—were compared by histological examination before and after extraction.

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MATERIALS & METHODS

A FREEZE-DRIED center corium split from a limed beef hide prepared by the method described by Komanowsky et al. (1974) was used for reference collagen. Rabbit actomyosin and bovine neck ligament elastin were obtained from Sigma Chemical Corporation. The rabbit actomyosin was dialyzed to remove glycerol and potassium chloride before analysis.

Isolation of connective-tissue samples

The connective tissues used in these studies were carefully removed as epimysium, perimysium and endomysium from beef chuck and round beef cuts from eight animals at a local abattoir.

Epimysium, a sheath-like structure which surrounds each muscle, was removed manually from the surface. Both thick and thin pieces were studied because of obvious differences in texture and appearance. Perimysium, which is contiguous with epimysium and surrounds each fascicle of muscle fibers, was carefully removed from the chuck area of a single cut of beef. It was considered as a multicomponent sample since it contains endomysium, because of the manner by which it was obtained. Endomysium, which is contiguous with the perimysium and surrounds each muscle fiber within the fascicle, was carefully removed from chuck under a dissecting microscope.

All isolated connective tissues were first soaked in deionized water to remove water-soluble material or blood. Connective tissues were subjected to extensive extractions of a chloroform/methanol mixture (3:1, v/v) to dissolve associated fat. Connective tissues were cycled through several changes of deionized water to remove all traces of solvent and blotted dry with paper towels prior to moisture determination and salt extraction.

Salt extractions

After lipid extraction, separate portions of connective tissue or meat samples in the wet blotted state were weighed, and dry weights were calculated from moisture determinations (*vide infra*). The samples were extracted with increasing molarities of potassium chloride (0.15M, 0.45M, 0.75M and 1.00M) for 2 hr each at 25°C. This removed sarcoplasmic proteins, soluble proteins including soluble collagen, and some myofibrillar proteins. The salt-extracted connective tissue was washed thoroughly with deionized water before lime extraction. Before the salt extracts were analyzed, the wash solutions and extracts were combined and dialyzed extensively to remove potassium chloride. The protein material remaining after dialysis was evaporated to dryness using a rotary evaporator prior to hydrolysis and amino-acid analysis.

Glycoprotein extraction

Connective tissue present after potassium chloride extraction and washing was extracted with half-saturated calcium hydroxide solution for 24 hr to remove glycoproteins (Eastoe and Eastoe, 1954). The residue from this extraction was washed with deionized water until washings were neutral, dried under vacuum, ground with a Wiley mill, and redried under vacuum prior to hydrolysis and amino-acid analysis. The same procedures were applied to chopped intact pieces of chuck and round beef.

After glycoprotein extraction, one set of samples was autoclaved for four 45-min periods in deionized water at 121°C and 15 psig (Partridge 1962). Extracts were evaporated to dryness, residues were air dried and ground with the Wiley mill. All samples were vacuum dried prior to amino-acid analyses.

Table 1—Amino-acid compositions of principal proteins in connective tissue

Amino acids	Collagen g/g	Elastin g/g	Actomyosin[a] g/g
Hydroxyproline	0.1129	0.0138	------
Aspartic Acid	0.0583	0.0093	0.1007
Threonine	0.0181	0.0103	0.0500
Serine	0.0313	0.0092	0.0407
Glutamic Acid	0.1016	0.0254	0.1916
Proline	0.1383	0.1378	0.0303
Glycine	0.2155	0.2173	0.0279
Alanine	0.0809	0.1947	0.0556
Cystine	------	------	0.0206
Valine	0.0234	0.1596	0.0482
Methionine	0.0084	0.0012	0.0362
Isoleucine	0.0147	0.0331	0.0519
Leucine	0.0302	0.0815	0.0876
Tyrosine	0.0081	0.0173	0.0409
Phenylanine	0.0214	0.0535	0.0420
Isodesmosine	------	0.0041	------
Desmosine	------	0.0100	------
Histidine	0.0071	0.0017	0.0228
Hydroxylysine	0.0106	------	------
Lysine	0.0362	0.0071	0.0983
Arginine	0.0829	0.0127	0.0690
	100g AA/100g AA residues		

[a] Beef actomysin calculated on basis of 69% myosin and 31% actin.

Amino acid analysis

Protein samples were hydrolyzed with constant boiling hydrochloric acid under nitrogen for 24 hr. We found no loss of amino acids under these conditions. The acid was evaporated, and the samples were made up to volume in 0.1 N HCl. Analyses were done with a 119CL Beckman amino-acid analyzer at 60°C, using a 180-min single column (Fauconnet and Rochemont, 1978). All analyses were done in duplicate.

Moisture determination

An aliquot of the connective-tissue sample was weighed accurately, dried in a vacuum oven 48 hr at 50°C at less than 5 torr, and reweighed. The sample was then ground using a semimicro Wiley mill and passed through a #10 mesh screen. It was redried in the vacuum oven and weighed prior to hydrolysis and amino-acid analysis.

Histology

Connective tissue samples taken before and after the extractions with potassium chloride and calcium hydroxide and after autoclaving were fixed in 10% neutral formalin and prepared for histological evaluation. Sections were cut on a Spencer freezing microtome at 30 to 50 μm. The following stains were used for identification of specific elements: Weigert for elastin, Van Gieson (McClung, 1950) or picro-Sirius red F3BA (Sweat et al., 1964) for collagen and muscle, and Sudan IV or Oil Red O (Lillie, 1965) for fat identification and distribution. Toluidine blue (Johnson, 1968), a metachromatic stain, was used for identification of glycoproteins. Photomicrographs were made with a Zeiss Photomicroscope.

RESULTS & DISCUSSION

Amino-acid analyses of the principal proteins are shown in Table 1. Because purified beef actomyosin was unavailable, amounts of amino acids in beef actomyosin in Table 1 were calculated from our determination of rabbit actomyosin, using data of Bodwell and McClain (1978). Since the amino-acid profiles of actomyosin, collagen, and elastin differed markedly, the amounts of the three most variable amino acids could be used in three simultaneous equations to estimate the levels of these three proteins:

$$\begin{aligned} \text{Wt-\% Hydroxyproline} &= 0.1129C + 0.0138E + 0.0A \\ \text{Wt-\% Valine} &= 0.0234C + 0.1596E + 0.0482A \\ \text{Wt-\% Glutamic acid} &= 0.1016C + 0.0254E + 0.1916A \end{aligned} \quad (1)$$

Collagen has a large amount of hydroxyproline (11.29%, weight basis); elastin has little; and actomyosin has none. Similarly, elastin has a high valine content (15.96%) and actomyosin has a high glutamic acid content (19.16%). In the calculations the weight percentages of actomyosin (A), collagen (C), and elastin (E) in connective-tissue samples after all solubilized constituents had been removed were determined from Equation 1 and normalized so that they sum to 100%. Desmosine and isodesmosine were two amino acids found in small quantities in elastins from *ligamentum nuchae* and aortas (Partridge et al., 1963; Miller et al., 1964). Although their determination is possible, they were not used to determine elastin because of the small amounts present.

Table 2—Amino-acid composition of dissected connective tissue and chopped whole beef. Values calculated from protein (collagen, elastin, actomyosin) compositions determined here were compared with direct experimental values

	Connective tissue from chuck %		Chopped chuck roast %		Connective tissue from round roast %		Chopped round roast %	
	Calculated	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated	Experimental
Hydroxyproline	5.714	5.681	0.721	0.698	6.299	6.194	1.946	1.884
Aspartic Acid	6.799	7.601	9.252	9.568	5.710	6.482	8.330	9.162
Threonine	2.916	2.993	4.569	4.543	2.395	2.695	4.032	4.158
Serine	3.196	3.493	3.812	3.850	2.844	3.104	3.553	3.667
Glutamic Acid	12.570	12.496	17.600	17.050	10.551	10.377	15.796	15.293
Proline	9.728	8.457	4.319	3.763	11.235	10.347	5.993	3.982
Glycine	14.467	13.001	3.952	4.374	17.104	15.184	7.968	7.053
Alanine	8.623	7.186	6.585	6.118	10.116	9.421	7.585	6.660
Cystine	------	------	------	------	0.049	------	------	------
Valine	5.063	5.035	5.388	5.220	6.083	5.982	5.730	5.547
Methionine	1.800	1.818	3.311	3.034	1.340	1.447	2.772	2.795
Isoleucine	3.129	3.414	4.862	5.027	2.785	3.139	4.382	4.822
Leucine	5.863	6.261	8.390	8.826	5.576	6.043	7.774	8.408
Tyrosine	2.173	2.468	3.756	4.003	1.807	2.195	3.296	3.639
Phenylanine	3.340	3.414	4.157	4.450	3.373	3.687	4.008	4.288
Isodesmosine	------	------	------	------	0.957	0.262	------	------
Desmosine	------	------	------	------	0.232	0.288	------	------
Histidine	1.232	1.657	2.058	2.607	0.961	1.274	1.785	2.214
Hydroxylysine	------	------	------	------	------	------	0.167	0.211
Lysine	5.588	6.012	8.902	9.568	4.430	4.731	7.783	8.624
Arginine	6.844	8.362	6.621	7.554	6.336	6.626	6.463	7.655
Protein	Collagen 49.01% Elastin 13.11 Actomyosin 37.88		Collagen 5.61% Elastin 6.35 Actomyosin 88.04		Collagen 52.96% Elastin 23.12 Actomyosin 23.92		Collagen 15.81% Elastin 11.68 Actomyosin 72.51	
Correlation	1.045		0.9980		0.9984		0.9936	

Table 3—Correlations obtained between the calculated amino-acid profile of connective tissue (with actomyosin) from chuck roast (Table 2) and the profile calculated for this connective tissue contaminated with various fractions of albumin

Albumin fraction	Correlation
0.240	0.98332
0.220	0.98605
0.200	0.98853
0.180	0.99076
0.160	0.99274
0.140	0.99448
0.120	0.99597
0.100	0.99722
0.080	0.99823
0.060	0.99901

Table 4—Amino-acid composition of protein extracts

Amino acid	g/100g protein	
	Combined salt extracts	Glycoprotein extract
Methionine sulfoxide	0.21	0.35
Hydroxyproline	N.D.[a]	N.D.
Aspartic acid	9.78	10.48
Threonine	4.51	4.05
Serine	4.09	4.18
Glutamic acid	17.12	15.33
Proline	4.58	4.33
Glycine	4.21	3.80
Alanine	6.58	5.44
Cystine	1.41	0.11
Valine	5.88	5.66
Methionine	1.55	1.89
Isoleucine	3.64	3.86
Leucine	8.97	9.01
Tyrosine	3.13	3.63
Phenylanine	4.35	5.16
Glucosamine	N.D.	2.04
Galactosamine	N.D.	1.84
Histidine	2.83	3.73
Hydroxylysine	N.D.	N.D.
Lysine	10.87	9.69
Arginine	5.77	5.44

[a] N.D. = Not detected levels are below 0.3 mg/500 mg protein.

Table 5—Comparison of proteins in various connective tissues

Sample analyzed	% Actomyosin	% Collagen	% Elastin
Perimysium (multicomponent sample)	28.8	58.2	13.0
Thin epimysium	18.8	73.8	7.4
Thick epimysium	27.4	61.7	10.9
Endomysium	79.5	20.5	0

Equation 1 was used to estimate relative amounts of collagen, elastin and actomyosin in connective tissue and whole beef. These calculated values of and their respective amino-acid profiles (Table 1) were used to calculate amino acid contents of the tissues listed in Table 2.

The correlations between the calculated and experimental amino-acid profiles of the samples appear at the bottom of Table 2. These are defined as

$$\frac{\Sigma\, a_i\, b_i}{(\Sigma\, a_i^2)^{1/2}\,(\Sigma\, b_i^2)^{1/2}}$$

where a_i = calculated amount of amino acid i and b_i = determined amount. The correlation can vary from 1 (perfect agreement) to 0 (peptide with only one amino acid correlated with one that does not contain that amino acid). The significance of these correlations is shown in Table 3. The calculated amino-acid profile for connective tissue from chuck (Table 2) correlated with profiles calculated for various mixtures of that connective tissue composition with albumin. Values of the correlations for connective tissue from chuck and from round roast, 1.040 and 0.9984 respectively, correspond to levels of contaminant albumin that were too low to detect. From tables similar to Table 3 for the chopped meat samples, we found the correlations for chopped chuck and chopped round, which had been drastically extracted, but from which connective tissue had not been dissected, corresponded to 14% and 20% albumin, respectively. That was because albumin and the apparent major protein actomyosin had relatively similar amino-acid profiles. Therefore the residue from extraction of this meat that was neither collagen nor elastin was not pure actomyosin, but contained other proteins as well. This type correlation analysis would be necessary for confidence in results of this method especially if connective tissue had not been obtained by dissection.

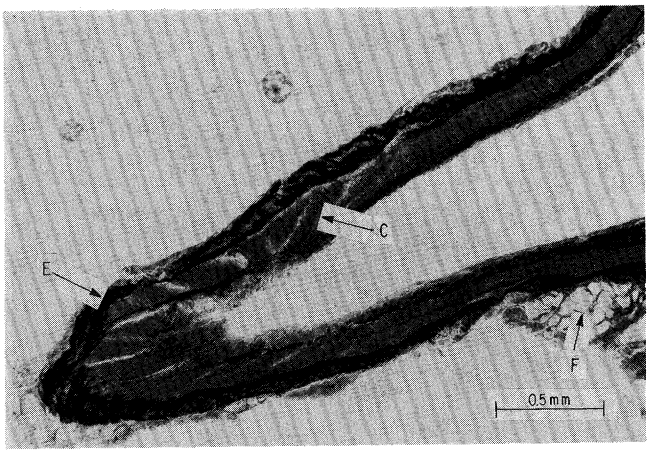

Fig. 1—Epimysium, showing collagen (C), elastin (E), and fat cells (F). Stained with Weigert and Van Gieson stains.

The results of sequential salt extractions of dissected connective tissue from chuck showed only 0.76% removal of starting material, indicating excellent isolation from the meat. The calcium hydroxide extraction removed 2.83%, the amount expected from the glycoprotein content. Glucosamine and galactosamine, each about 2% on a weight basis, were present in hydrolysates of the glycoprotein fraction, as expected from their presence in the glycosaminoglycan moiety of some glycoproteins. Amino acid analyses of the extracts are listed in Table 4. Collagen and probably elastin were absent, since hydroxyproline was not detected in the extracts. The amino-acid profiles of these lime extracts resembled that of the mucopolysaccharide extracted by Eastoe and Eastoe (1954). However, the lysine contents of our samples were twice those found in their analysis.

Table 5 shows protein content of the different types of connective tissues studied. Analyses showed that the dissected endomysium was probably highly contaminated with actomyosin; nevertheless the endomysium would seem to contribute little to toughness since it has no elastin, and a significantly lower amount of collagen was found in it than in the perimysium or in the epimysium. Under the microscope, muscle fibers were not evident in this tissue.

The presence of elastin in the epimysium and perimysium was shown by amino-acid analyses and histological procedures. Microscopy showed major differences in distribution of elastin in these tissues. It was uneven and random in the epimysium, less random and chiefly in the arterial systems of the perimysium, and essentially absent from the endomysium (Fig. 1, 2 and 3). The metachromatic effects of staining connective tissue with toluidine blue, indicating presence of glycoproteins associated with connective tissue, is shown in Fig. 4. Muscle tissue is shown at the top and bottom of the figure. After removal of the glycoproteins by the calcium hydroxide treatment, the metachromatic effect of toluidine blue is lessened, and this is also shown by the amino-acid analysis of the hydrolyzed alkaline extract (Table 4).

The fate of the connective tissues during autoclaving may be related to the cooking of meat. Table 6 shows that all the collagen was solubilized during the sequential autoclavings.

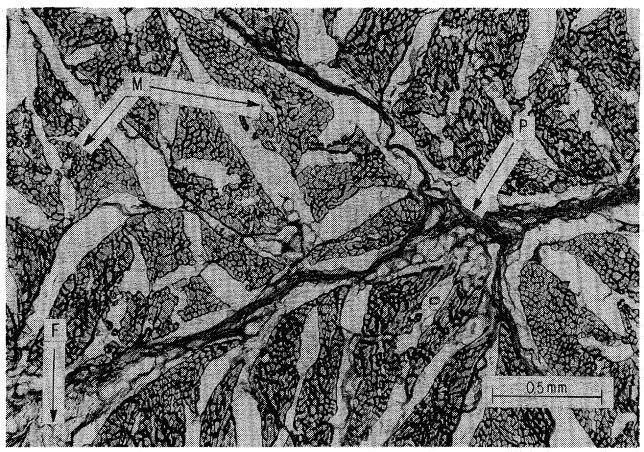

Fig. 2—Cross-section of meat from chuck, showing perimysium (P), muscle (M), and fat cells (F). Stained with picro-Sirius red.

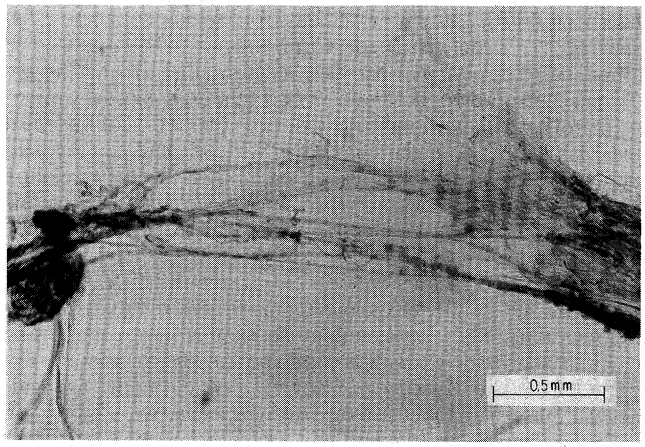

Fig. 3—Endomysium teased from muscle cells. Stained with Weigert and Van Gieson stains.

Most was removed during the first autoclaving, which extracted most of the heat soluble material, 43.7% as compared to 19.3% total for the second, third, and fourth extracts. Elastin in connective tissues was most resistant to this type heat treatment.

Meat with a high connective-tissue content is often treated with enzymes and other chemicals to degrade or soften these tissues and make the meat more palatable. The fractionation procedures and method described here help determine amounts of actomyosin, collagen, and elastin in connective-tissue samples. This should be useful in confirming feasibility and specificity of experimental treatments to selectivity remove or degrade specific connective tissue components.

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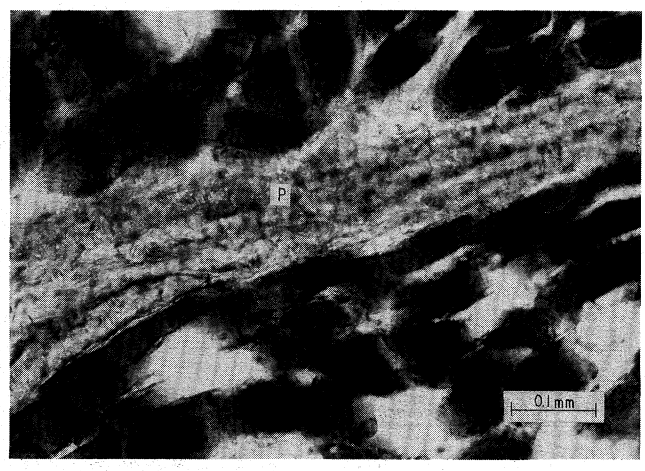

Fig. 4—Cross-section of meat showing perimysium (P) stained with Toluidine blue to demonstrate the presence of glycoproteins.

Table 6—Heat soluble proteins from autoclaved chuck connective tissue

Autoclave cycles	Proteins in extract (%)			Weight (%) of starting material
	Collagen	Elastin	Actomyosin	
1	95.1	0	4.9	43.7
2	89.0	1.1	9.9	15.1
3	46.1	5.0	48.9	2.9
4	14.9	8.3	76.8	1.3
Final residue	0	68.3	31.4	37.1

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